CHAPTER 3

EXPRESSION OF APOPTOSIS-RELATED PROTEINS IN BARRETT'S METAPLASIA-DYSPLASIA-CARCINOMA SEQUENCE: A SWITCH TO A MORE RESISTANT PHENOTYPE

C.J. van der Woude, P.L.M Jansen, A.T.G.M. Tiebosch, A. Beuving, M. Homan, J.H. Kleibeuker, H. Moshage

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SUMMARY

Barrett's esophagus (colomnar-lined esophagus, CLE) is a pre-malignant disorder in which the stratified squamous epithelium is replaced by metaplastic epithelium. To gain more insight into the process of carcinogenesis in CLE we studied several factors involved in the apoptotic pathway in biopsies with gastric metaplasia (GM), intestinal metaplasia (IM), dysplasia, and/or adenocarcinoma. Immunohistochemistry was performed for Fas, Bcl-2 , Bax , Bcl-xl, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Fas staining was positive in epithelium of all biopsies from patients with CLE but negative in normal gastric mucosa. Fas staining was positive in all tumor cells of the 8 cases containing adenocarcinoma. Bcl-2 was positive in lamina propria immune cells of all specimens. Bax staining was positive in the epithelium of all biopsies, including tumor cells. Bcl-xl was positive in dysplasia and tumor cells, but negative in 8 of 17 biopsies containing IM. iNOS was positive in 20 of 21 biopsies with IM and in 4 of 8 dysplasia biopsies. COX-2 was positive in 7 of 8 adenocarcinomas. We conclude that the apoptotic balance in the transformation from IM to adenocarcinoma switches to an anti-apoptotic phenotype because of increased Bcl-xl expression and decreased Bax expression. Fas can be used as a marker for the differentiation of gastric mucosa and metaplasia in the esophagus. iNOS is highly positive in CLE-associated intestinal metaplasia. COX-2 negative in non-malignant CLE. Therefore pharmacological inhibition of COX-2 activity is unlikely to be effective in the prevention of CLE-associated adenocarcinoma. There was no clear correlation between iNOS expression and activation of pro- and anti-apoptotic genes.

1. INTRODUCTION

In Barrett's esophagus, or colomnar-lined esophagus (CLE), the normal stratified squamous epithelium lining the esophagus has been replaced by metaplastic columnar epithelium containing goblet cells[1]. This replacement is a risk factor for neoplastic transformation, and there is evidence for the sequential development of adenocarcinoma via intestinal metaplasia and low grade and high grade dysplasia[2,3] Therefore, periodic surveillance endoscopy with multiple biopsies is recommended for CLE patients. Other modalities to evaluate the esophagus for Barrett's metaplasia

and impending malignant degeneration have been investigated, but histologic examination remains the gold standard[4-7]. To simplify surveillance new preventive treatment options are needed[8-12].

Disturbances in apoptosis are supposed to play an important role in the sequential development of dysplasia and cancer. To gain better insight in these disturbances we studied the expression of 4 apoptosis-related proteins Fas, Bcl-2, Bax and Bcl-xl. We further studied the expression of 2 other closely apoptosis-related proteins: inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Nitric oxide, produced by iNOS, has been demonstrated to inhibit apoptosis by inhibiting caspase activity. However, chronic exposure to high levels of nitric oxide can also promote apoptosis[13].

High COX-2 expression has been demonstrated in human colorectal adenomas and in gastric adenocarcinomas[14,15]. Inhibition of COX-2 activity promotes apoptosis and could be a promising modality for chemoprevention of these tumors, as has been shown in patients with familial adenomatous polyposis[16,17].

2. MATERIALS AND METHODS

2.1 Patient selection and tissue collection

We studied tissue samples taken from patients who participated in an endoscopy surveillance program between January 1998 and December 2000 at the University Hospital Groningen. All patients used omeprazole at the time of endoscopy. Samples were stained with haematoxylin and eosin and periodic acid-Schiff. Standard histological examination was performed on these stained samples with attention given to the type of metaplasia, presence and degree of inflammation and dysplasia and presence of adenocarcinoma. The most distal samples from the esophagus from each patient were used in this study. Samples from patients with esophagitis graded according to Savary and Miller[18] during endoscopy and from those with histologically active inflammation, graded according to Paull et al[19], were excluded from this study. After exclusion of these samples and samples without metaplasia, dysplasia or adenocarcinoma, the samples were histologically graded by a single pathologist. Samples were categorized as GM or specialized columnar metaplasia[19]. Dysplasia was scored as absent, indefinite-low grade and high grade[20]. Different histological gradations could coexist in one sample.

2.2 Immunohistochemical analysis

All stainings were performed on deparaffinized 4 micrometer thick sections. These sections were cut from formalin-fixed and paraffin embedded tissues.

An overview of the methods is given in table 1.

Table 1: Immunohistochemistry methods

Protein	Section	Antigen retrieval	Primary antibody
Fas	Paraffin	2x15min at 98°C in 1mM EDTA; pH6.0	Mouse monoclonal at 1:400 Upstate Biotechnology, Lake Placid; cat. nr. 05-201
Bcl-xl	Paraffin	2x15min at 98°C in 0,1M Tris HCl; pH9.0	Mouse monoclonal at 1:100 Zymed Laboratories, South San Francisco; cat. nr. 33-6300
Bax	Paraffin	MW (700W) 8 min in 10mM citrate; pH6.0	Mouse monoclonal at 1:400 Santa Cruz Biotecnology P19, Santa Cruz ; cat. nr. SC-526
Bcl-2	Paraffin	MW (700W) 8 min in 10mM citrate; pH6.0	Mouse monoclonal at 1:50 Dako Glostrup, Denmark; cat. nr. M 0887
COX-2	Paraffin	2x15min at 98°C in 1mM EDTA; pH6.0	Mouse monoclonal at 1:50 Transduction Laboratories;cat. nr. C-22420
iNOS	Paraffin	2x15min at 98°C in 1mM EDTA; pH6.0	Mouse monoclonal at 1:50 Transduction Laboratories: cat. nr. N-39120

2.3 Quantitation of immunoreactivity

The immunohistochemical sections stained with Fas, Bcl-2, Bax, Bcl-xl, iNOS and COX-2 were scored by 3 different observers for the percentages of epithelial cells stained. In case of differences in interpretation, the sample was scored again, and a consensus was reached. Absence of staining was scored as 0; 0 to 10% staining was scored as 1; 11 to 50% staining as 2; and 51 to 100% was scored as 3. Tissue samples stained with Fas, Bcl-xl and Bax were also scored for intensity of staining in each individual epithelial cell on a scale of 0 to 3, with 0 being negative; 1, weak; 2, moderate; 3, strong.

Staining of tumor cells was scored in the same way.

2.4 Statistical analysis

The relationship between grade and intensity in expression of each separate protein on the one hand and the histological parameters on the other was evaluated using Somers'd test was performed. The analysis was performed using Sigmaplot Scientific Software (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was considered significant.

3. RESULTS

3.1 Patients

Samples from 28 patients (20 male, 8 female) were included. The age of these patients was 31 to 86 years (mean 58). Six samples contained gastric metaplasia (GM), 21 contained intestinal metaplasia (IM), 8 contained indefinite for and low grade dysplasia (D) and 6 contained high grade dysplasia and carcinoma (CA). The adenocarcinoma group was expanded with archival resection material from 4 patients with CLE-associated adenocarcinoma.

3.2 Immunohistochemistry (table 2 and 3)

3.2.1 Fas staining in epithelium and tumor cells

Three of 21 tissue samples containing IM, 2 of 8 samples containing dysplasia and 1 of 10 samples containing CA were not stained, because of a lack of material. Fas staining was present in the epithelium of all tissue samples, including GM (fig. 1a). Tumor cells were all positive (fig. 1b). Fas staining of normal gastric mucosa in patients with GM and in controls was negative (fig. 1c). The correlation between staining grade and sequence from IM to CA was significant: r = 0.527 (± 0.126), p= 0.01. The correlation between staining intensity and sequence from IM to CA was also significant: r = 0.329 (± 0.126), p < 0.001.

Table 2 . Staining *intensity* of Fas, Bax and Bcl-xl expression

Intensity	Fas*				Bax**				Bcl-xl***			
	GM	IM	D	CA	GM	IM	D	CA	GM****	IM	D	CA
0	0	1	0	0	0	0	0	1		8	0	1
1	0	4	2	0	1	4	4	6		8	1	0
2	3	9	2	3	1	7	2	2		1	4	3
3	3	4	2	6	3	9	2	0		0	3	6

* a significant difference from intestinal metaplasia to cancer, ** a significant difference from intestinal metaplasia to cancer
*** a significant negative difference from intestinal metaplasia to cancer, **** not scored

Table 3. Staining *grade* of iNOS, Fas, Bax and Bcl-xl expression

Grade	iNOS*				Fas**				Bax***				Bcl-xl****			
	GM	IM	D	CA	GM	IM	D	CA	GM	IM	D	CA	GM*****	IM	D	CA
0	6	1	4	6	0	1	0	0	0	0	0	1		8	0	1
1	0	3	4	0	0	5	2	0	0	3	0	0		7	1	0
2	0	14	0	0	2	9	1	0	2	3	0	0		2	3	2
3	0	3	0	0	4	3	3	9	3	14	8	8		0	4	7

* significant negative difference from intestinal metaplasia to cancer, ** significant difference from intestinal metaplasia to cancer
*** not significant difference from intestinal metaplasia to cancer, **** significant difference from intestinal metaplasia to cancer
***** not scored

3.2.2 Bcl-2 staining in epithelium and tumor cells

Bcl-2 staining was negative in epithelium of CLE and was also not present in tumor cells. Lamina propria immune cells showed positive staining (fig. 1d).

3.2.3 Bax staining in epithelium and tumor cells

Because of a lack of material 1 tissue sample of the GM, one of the IM group and one of the CA group was excluded from the Bax staining series. In all groups, epithelial cells stained positive (fig. 1e). In tumor cells, Bax staining was also clearly positive (fig. 1f).

The correlation between staining grade and sequence from IM to CA was not significant: $r = 0.302$ (± 0.197), $p = 0.141$. The correlation between staining intensity in each individual epithelial cell and sequence from IM to CA was significant.
$r = –0.443$ (± 0.101), $p = 0.001$.

3.2.4 Bcl-xl staining in epithelium and tumor cells

Because of a lack of material, 4 tissue samples of the IM group were excluded from the Bcl-xl staining series. Staining in dysplasia and tumor cells (fig. 1g) was mostly positive with a strong intensity. The correlation between staining grade and sequence from IM to CA was significant: $r = 0.600$ (± 0.085), $p < 0.001$. The correlation between staining intensity and sequence was also significant: r = 0.600 (± 0.088), $p < 0.001$.

3.2.5 iNOS staining in epithelium and tumor cells

iNOS staining was intensely positive in epithelium of IM (fig. 1h). Epithelial staining was positive in 4 out of 8 samples containing dysplasia (fig. 1i). GM and tumor cells

were negative for iNOS. The correlation between staining grade and sequence from IM to CA was significant: $r = –0.678$ (± 0.084), $p < 0.001$.

3.2.6 COX-2 staining in epithelium and tumor cells

COX-2 expression was not present in epithelium of CLE and associated dysplasia. Lamina propria immune cells and myofibroblasts showed positive staining (fig. 1j). In the adenocarcinoma group, tumor cells, but not normal epithelium, were positive for COX-2 in 9 of 10 samples. In these biopsies however, only a minority of tumor cells stained positive (fig. 1k).

4. DISCUSSION

In CLE, iNOS is highly expressed in IM and in 50% of samples containing dysplasia, but not in CLE associated adenocarcinoma. All our samples containing high-grade dysplasia were positive for iNOS, as reported by Wilson et al[21]. However, in contrast to these authors, we did not observe iNOS expression in CLE-associated adenocarcinomas. Nitric oxide, the product of iNOS, is able to inhibit apoptosis in low concentrations, due in part to inhibition of caspase activity[13]. In high concentrations, it can induce apoptosis. We could not detect apoptosis in CLE intestinal metaplasia using staining for caspase-cleaved cytokeratin 18 (cytodeath) (data not shown). Whether this means that iNOS inhibits apoptosis in CLE IM remains to be established, because apoptosis was also absent in iNOS negative CLE dysplasia.

The role of apoptosis in the sequence of IM to adenocarcinoma is not clear[22,23]. Our results suggest that Bcl-2 is not involved in the carcinogenesis of CLE, because only lamina propria immune cells, not the epithelium, showed positive staining. Bax, a pro-apoptotic member of the Bcl-2 family, was positive in all samples. Although no significant differences in staining grade was observed among the different groups, there was a significant negative correlation between intensity of Bax staining in each individual epithelial cell and the transformation of IM to adenocarcinoma. According to these observations, the epithelial cells transform into less Bax-positive cells and thus more apoptosis-resistant cells. These results contrast with previous reports[24] that found a positive association between progression to adenocarcinoma in CLE and Bax expression.

Members of the Bcl-2 family play an important role in the regulation of apoptosis. This family contains pro-apoptotic members (Bax, Bid, Bad, Bak) and anti-apoptotic members (Bcl-2, Bcl-xl). Bcl-2 proteins regulate the permeability of the mitochondrial membrane. Increased mitochondrial permeability allows leakage of cytochrome C from mitochondria into cytoplasm, triggering caspase activation and apoptosis. Proapoptotic Bcl-2 proteins increase mitochondrial membrane permeability, whereas antiapoptotic members antagonize the effects of proapoptotic Bcl-2 proteins[25]. The continuous expression of Bax could be triggered by overexpression of mutated p53 (proapoptotic) found in earlier reports[26,27].

Bcl-xl, an antiapoptotic Bcl-2 family member, was highly positive in dysplasia and tumor cells but not in IM. The increase in Bcl-xl grade and intensity of staining in the transformation from IM to CLE-associated adenocarcinoma was significant. The reciprocal changes in the expression of Bax and Bcl-xl in the sequence from IM to adenocarcinoma indicate that these cells become increasingly more resistant to apoptotic cell death, giving these cells a survival and proliferation advantage.

Fas is a member of the tumor necrosis factor receptor superfamily. Activation of this receptor by its ligand activates caspase 8 and the apoptotic signal transduction pathway. Fas expressing cells are vulnerable to Fas ligand induced cell death. Fas ligand is predominantly expressed by lymphocytes, but can also be expressed by other cells. Therefore, Fas-mediated cell death can occur only when Fas ligand-positive cells are in close proximity to Fas-positive target cells[28]. Fas was not only present in CLE IM, but also in GM of the esophagus. Decreased Fas expression has been reported in CLE. However, in this study Fas staining of goblet cells was investigated[29]. Previously, Fas expression was not found in normal gastric mucosa[30] and we confirmed these results. Therefore, Fas expression can be used to differentiate between normal gastric mucosa and GM in the esophagus. The expression of Fas ligand has been reported during malignant transformation of Barrett's metaplasia[31]. However, the simultaneous expression of Fas and Fas ligand does not necessarily lead to apoptotic cell death. Various antiapoptotic mechanisms may exist in Fas/Fas ligand co-expressing cells that protect these cells against apoptosis[32].

Most CLE-associated adenocarcinomas were COX-2 positive but only in a minority of tumor cells. In IM and dysplasia, COX-2 staining was negative and only lamina propria immune cells showed COX-2 expression. This contrasts with previous

reports[33,34], although other reports support our findings[35]. Pharmacological inhibition of COX-2 activity has been proven effective in reducing colonic polyp formation in humans. COX-2 staining in this study was negative in the pre-cancerous state in CLE. Our results do not support a role for COX-2 inhibition in the prevention or treatment of Barrett's dysplasia and cancer and a recent report from Tsibouris et al found no differences in cancer occurence in CLE in the presence or absence of nonsteroidal anti-inflammatory drugs[36].

All patients in our study were using proton-pump inhibitors (PPI). Peters et al reported that high dose PPI treatment resulted in partial endoscopic regression of CLE,[37] and effective PPI treatment decreased proliferation in an earlier study[38]. However other factors, such as duodenogastroesophageal reflux, may contribute to the development of CLE. Therefore, the effects of PPI treatment on proliferation in Barrett's esophagus remains unclear. Likewise, nothing is known about the effect of PPI treatment on apoptosis in Barrett's esophagus. Considering the regression of metaplasia and decreased proliferation in Barrett's esophagus of patients using PPIs, a proapoptotic effect of PPI could be hypothesized, but data are lacking.

In conclusion, the apoptotic balance in the transformation from IM to adenocarcinoma switches to an antiapoptotic phenotype due to increased Bcl-xl expression and decreased Bax expression. Most Barrett's esophagus-associated adenocarcinomas are COX-2 positive but only in a minority of tumor cells. COX-2 is not positive in non-malignant Barrett's esophagus. Therefore, pharmacological inhibition of COX-2 activity is unlikely to be effective in the prevention of Barrett's esophagus-associated adenocarcinomas. iNOS is highly positive in intestinal metaplasia and Fas expression can be used as a marker for differentation between normal gastric mucosa and gastric metaplasia in the esophagus.

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